PAPER n° 9

THE BEHAVIOUR OF *Alcaligenes denitrificans* IN DIFFERENT RESPIRATORY CONDITIONS: EFFECT OF C/N RATIO AND P CONCENTRATION

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ABSTRACT
In order to improve the rate of denitrification by *Alcaligenes denitrificans* the effect of phosphorous concentration and the carbon /nitrogen ratio, under aerobic and anoxic conditions, were studied. Batch experiments were conducted under anoxic and aerobic conditions, with two different carbon/nitrogen ratios (C/N = 2 and C/N = 3) and with three different phosphorous concentrations (P = 2 mg/l, P = 20 mg/l and P = 200 mg/l). In anoxic conditions, in all the experiments, *Alcaligenes denitrificans* showed a typical diauxic growth. The specific consumption rate of nitrate was very similar for both C/N ratios and for the three phosphorous concentrations studied. The consumption of citrate was almost the same for both C/N ratios, but increased with increasing phosphorous concentration. Moreover, for C/N = 2 citrate was completely consumed, while for C/N = 3, only about half of its concentration was consumed. This reveals that a C/N = 3 is too high.
In aerobic conditions, for both C/N ratios, citrate was always totally consumed while only a small amount of nitrate was consumed. Besides, there was no nitrite production. So, it seems evident that, in aerobic conditions, the substrates were only consumed for cell synthesis and no significant denitrification was observed.

KEYWORDS
Alcaligenes denitrificans; C/N ratio; denitrification; phosphorous concentration

INTRODUCTION

Generally denitrification is considered to be an anoxic process, occurring in the presence of nitrate and the absence of molecular oxygen (Gayle *et al*., 1989). However, recently, several workers showed that, in certain species, denitrification could occur in the presence of oxygen (Robertson and Kuenen, 1984; Lloyd *et al*., 1987). Although aerobic denitrification tends to be slower than anoxic denitrification (Robertson and Kuenen, 1992), if an organism is capable of both this would presumably enhance its survival ability (Lloyd *et al*., 1987). Denitrification is classically considered to be a heterotrophic process conducted by microorganisms that require a reduced organic substrate for energy and cell synthesis. As heterotrophic denitrifier, *Alcaligenes denitrificans* can utilize a great variety of organic carbon sources, such as, acetate, propionate, butyrate, citrate, pyruvate, etc (Bergey's manual, 1984). However, in the last few years it became important to use carbon sources compatible with the standards imposed for drinking water. Thus, acetate and citrate are being increasingly used. Blasczcyk (1983) found that in a packed bed reactor working with acetate, denitrifying bacteria were dominated by *Pseudomonas aeruginosa*, which are pathogenic. So, in this work, citrate was chosen as carbon source. An important parameter influencing the denitrification reaction is the carbon/nitrogen ratio. In the case of citrate, the stoichiometric reaction gives a ratio equal to 1.86. The values of the C/N ratios used in this work are slightly higher than the theoretical one in order to

take into account the carbon requirement for the deoxygenation of the medium. Microbial growth is also regulated by the availability of phosphorous. So, it is of great importance to determine the optimal phosphorous concentration, in order to obtain a stable biomass leading to high denitrification efficiency. The objective of this study was to examine the conditions for improving the rate of denitrification by *Alcaligenes denitrificans.*

MATERIALS AND METHODS

Organisms and culture medium

The denitrifying bacteria used was a pure culture of *Alcaligenes denitrificans* ATCC 15173, grown in a medium containing 0.2448 g $C_6H_5Na_3O_7 \cdot 2H_2O$, 0.289 g KNO_3, 0.93 g K_2HPO_4, 0.18 g KH_2PO_4, 0.0242 g $NaMoO_4 \cdot 2H_2O$, 0.0056 g $FeSO_4 \cdot 7H_2O$, 0.00081 g $MnCl_2 \cdot 2H_2O$, 0.0515 g $CaCl_2 \cdot 2H_2O$ and 0.4092 g $MgSO_4 \cdot 7H_2O$ in 1 litre of distilled water. The cultures were grown in batch during 3 days, at 30ºC and orbital shaking at 150 rpm. To obtain the desired biomass concentration, cells were harvested by centrifugation (10 min at 5000 rpm). The concentration of the final cellular suspension was measured by optical density at 660 nm.

Denitrifying batch assays

The experiments were carried out under two carbon/nitrogen ratios (C/N = 2 and C/N = 3). In both situations, three phosphorous concentrations were studied (P = 2 mg/l, P = 20 mg/l and P = 200 mg/l). All the experiments were performed under aerobic as well as under anoxic conditions. The assays were carried out in 160-ml flasks, with 90 ml of the culture medium with the appropriate C/N ratio. KNO_3 was used as electron acceptor (50 mg $N\text{-}NO_3^-$/l) and sodium citrate, K_2HPO_4 and KH_2PO_4 were added in order to give the desired C/N and phosphorous concentration. Each flask was then inoculated with 2.5 ml of the stock cell suspension. Aerobic experiments were carried out in bottles with an atmospheric headspace, which were sealed with cotton caps in order to allow oxygen transfer. Anoxic conditions were ensured by flushing the bottles with helium and immediately sealing. All bottles were incubated at 30ºC and submitted to orbital shaking of 150 rpm. All assays were done in duplicate. Aliquots of 2 ml of each bottle were taken every hour and immediately analysed. Biomass was calculated by optical density at 660 nm. The samples were filtered through 0.2 µm syringe filters and the filtrates were used for nitrate, citrate and nitrite determinations. In the end of each experiment the liquid volume remaining was measured and was used to determine total and volatile solids.

Analytical Methods

Citrate and nitrate concentrations were measured by HPLC (Jasco) in an organic acids column (Chrompack, 300 mm x 6.5 mm). Nitrite was determined by a colorimetric method using N-(1-naphthyl)-ethylene-diamine, according to the Standard Methods of Analysis (APHA, 1992). Total solids (TS) and volatile solids (VS) were determined also in accordance with the Standard Methods of Analysis.

Kinetics

The maximum specific growth rate (μ) of *A. denitrificans* was calculated directly from biomass (X) evolution:

$$m = \frac{1}{[X]} \frac{d[X]}{dt}$$

The specific consumption rate (q_s) of nitrate and citrate were calculated from the following equation:

$$q_S = -\frac{1}{[X]}\frac{d[S]}{dt}$$

Where S is the substrate consumed (nitrate or citrate).

RESULTS AND DISCUSSION

A set of 24 denitrifying batch experiments was performed under aerobic conditions and 24 under anoxic conditions. In aerobic conditions, under C/N = 2, the biomass was in average 1.5 times higher than the initial biomass concentration, whereas under C/N = 3 it was almost 3 fold. For both C/N ratios the biomass increased with increasing phosphorous concentration (Figure 1A and B).

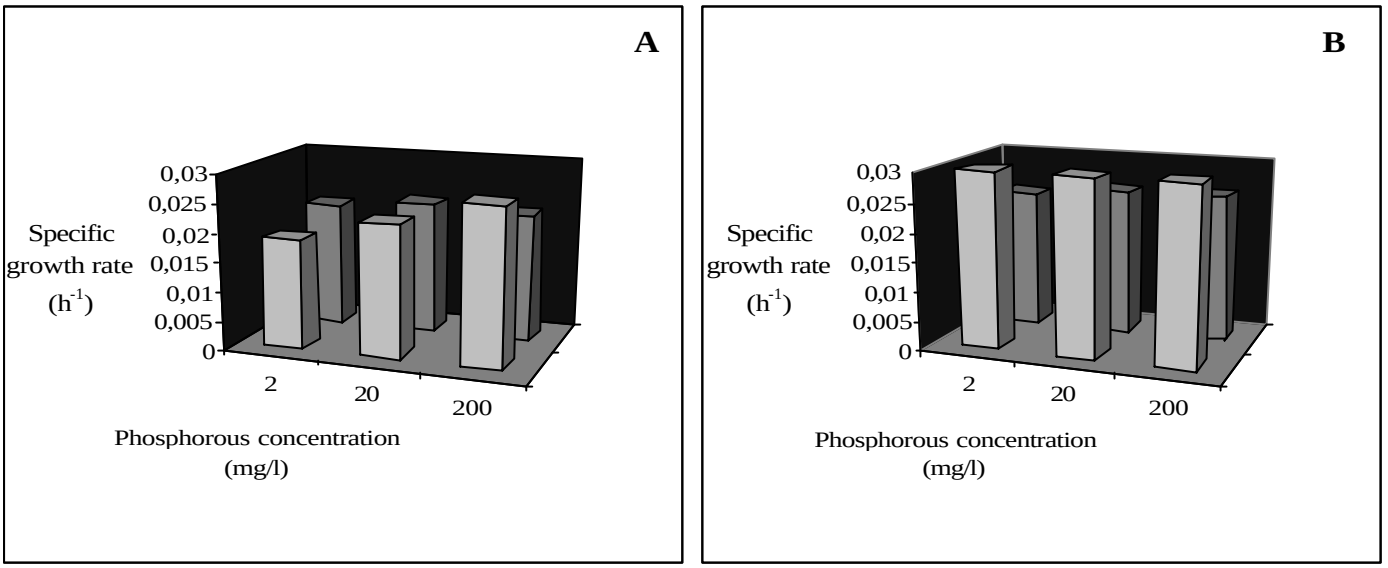

Figure 1. Effect of different phosphorous concentrations on the specific growth rate, under aerobic and anoxic conditions, for C/N = 2 (**A**) and C/N = 3 (**B**).

The nitrate reduction was in the range 20-37% and the specific nitrate consumption rate (gNO_3^-/h gbiomass) was always significantly lower than in anoxic conditions (Figure 2A and B). Citrate was always completely consumed and no nitrite accumulation was observed.

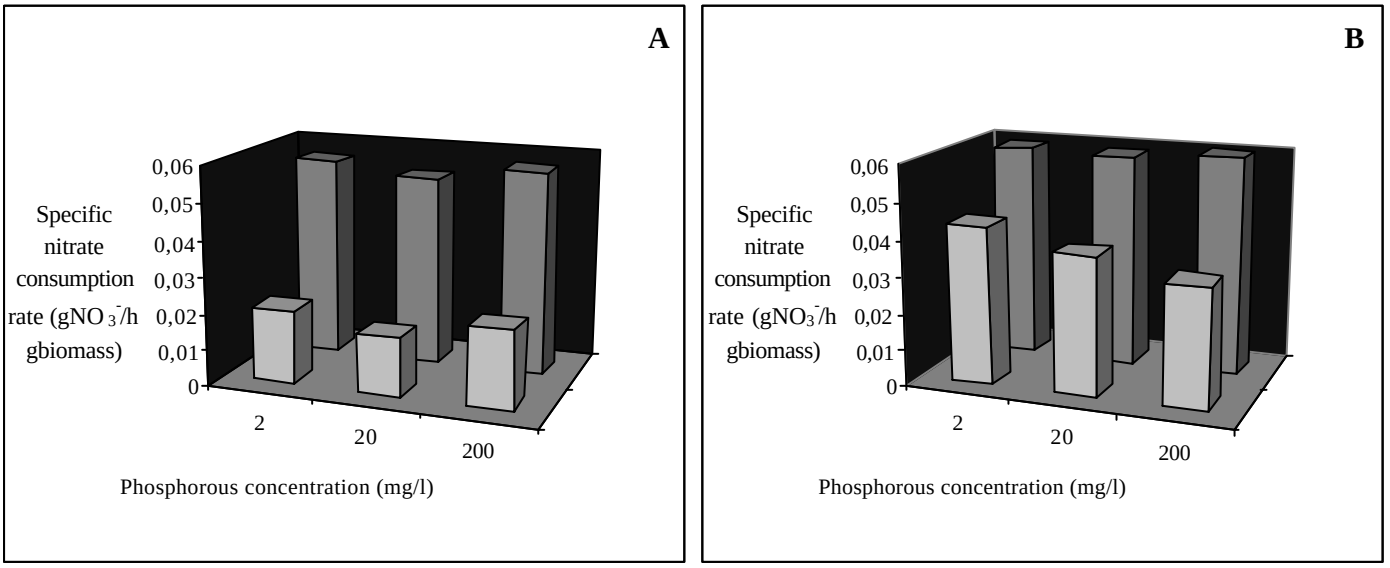

Figure 2. Effect of different phosphorous concentrations on the specific nitrate consumption rate, under aerobic and anoxic conditions, for C/N = 2 (**A**) and C/N = 3 (**B**).

Under anoxic conditions, *A. denitrificans* showed a typical diauxic growth: starting with nitrate reduction followed by reduction of nitrite after nitrate depletion. In these conditions the specific growth rate was very similar for all three phosphorous concentrations studied and, in generally, lower than under aerobic conditions. In anoxic tests, the specific nitrate consumption rate was very similar for both C/N ratios and

also almost independent of the phosphorous concentrations studied. For C/N =2, citrate was all consumed while for C/N = 3, only about half of its concentration was consumed, meaning that there was an over-addition of carbon. In these conditions, the specific consumption rate of citrate increased with the increase in phosphorous concentration, being always higher under aerobic conditions.

CONCLUSIONS

It seems that under aerobic conditions no denitrification occurred. There was only citrate consumption for biomass development and deoxygenation of the medium. The consumption of nitrate seems to have occurred only for cell synthesis. Under anoxic conditions, the specific consumption rates of nitrate for both C/N ratios were very similar, being slightly higher for C/N = 3. However, for this carbon/nitrogen ratio, there was always some remaining carbon, which is not desirable.

Regarding the effect of phosphorous concentration on the behaviour of *A. denitrificans*, it did not affect the specific growth rate under anoxic conditions as well as nitrate consumption.

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